

METHOD FOR PRODUCING ANTIBODY FRAGMENTSFIELD OF THE INVENTION

5 The present invention relates to an expression library comprising a repertoire of nucleic acid sequences cloned from a non-immunised source, each nucleic acid sequence encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains and its use in producing
10 antibodies, or more particularly fragments thereof. In particular, the invention relates to a method for the preparation of antibodies or fragments thereof having binding specificity for a target antigen which avoids the need for the donor previously to have been immunised with the target antigen.

BACKGROUND OF THE INVENTION

Monoclonal antibodies, or binding fragments thereof, have traditionally been prepared using hybridoma technology (Kohler and
20 Milstein, 1975, Nature 256, 495). More recently, the application of recombinant DNA methods to generating and expressing antibodies has found favour. In particular, interest has concentrated on combinatorial library techniques with the aim of utilising more efficiently the antibody repertoire.

25 The natural immune response *in vivo* generates antigen-specific antibodies via an antigen-driven recombination and selection process wherein the initial gene recombination mechanism generates low specificity, low-affinity antibodies. These clones can be
30 mutated further by antigen-driven hypermutation of the variable region genes to provide high specificity, high affinity antibodies.

Approaches to mimicking the first stage randomisation process
35 which have been described in the literature include those based on the construction of 'naive' combinatorial antibody libraries

prepared by isolating panels of immunoglobulin heavy chain variable (VH) domains and recombining these with panels of light variable chains (VL) domains (see, for example, Gram et al, Proc. Natl. Acad. Sa, USA, 89, 3576-3580, 1992). Naive libraries of antibody fragments have been constructed, for example, by cloning the rearranged V-genes from the IgM RNA of B cells of unimmunised donors isolated from peripheral blood lymphocytes, bone marrow or spleen cells (see, for example, Griffiths et al, EMBO Journal, 12(2), 725-734, 1993, Marks et al, J. Mol. Biol., 222, 581-597, 1991). Such libraries can be screened for antibodies against a range of different antigens.

In combinatorial libraries derived from a large number of VH genes and VL genes, the number of possible combinations is such that the likelihood that some of these newly formed combinations will exhibit antigen-specific binding activity is reasonably high provided that the final library size is sufficiently large. Given that the original B-cell pairing between antibody heavy and light chain, selected by the immune system according to their affinity of binding, are likely to be lost in the randomly, recombined repertoires, low affinity pairings would generally be expected. In line with expectations, low affinity antibody fragments (Fabs) with K_{as} of 10^4 - 10^5 M^{-1} for a progesterone-bovine serum albumin (BSA) conjugate have been isolated from a small (5×10^6) library constructed from the bone marrow of non-immunised adult mice (Gram et al, see above).

Antibody fragments of higher affinity (K_{as} of 10^6 - 10^7 M^{-1} range) were selected from a repertoire of 3×10^7 clones, made from the peripheral blood lymphocytes of two healthy human volunteers (Marks et al, see above) comprising heavy chain repertoires of the IgM (naive) class. These were combined with both Lamda and Kappa light chain sequences, isolated from the same source. Antibodies to more than 25 antigens were isolated from this library, including self-antigens (Griffiths et al, see above) and cell-surface molecules (Marks et al, Bio/Technology, 11, 1145-1149,

1993).

The second stage of the natural immune response, involving affinity maturation of the selected specificities by mutation and selection has been mimicked *in-vitro* using the technique of random point mutation in the V-genes and selecting mutants for improved affinity. Alternatively, the affinity of antibodies may be improved by the process of "chain shuffling", whereby a single heavy or light chain is recombined with a library of partner chains (Marks et al, Bio/Technology, 10 779-782, 1992).

Recently, the construction of a repertoire of 1.4×10^{10} scFv clones, achieved by 'brute force' cloning of rearranged V genes of all classes from 43 non-immunised human donors has been reported (Vaughan et al 1996) and Griffiths et al, see above. Antibodies to seven different targets (including toxic and immunosuppressant molecules) were isolated, with measured affinities all below 10nM.

The main limitation in the construction of combinatorial libraries is their size, which consequently limits their complexity. Evidence from the literature suggests that there is a direct link between library size and diversity and antibody specificity and affinity (see Vaughan et al, Nature Biotechnology, 14, 309-314, 1996), such that the larger (and more diverse) the library, the higher the affinity of the selected antibodies. On this basis, single domain libraries, which omit the process of recombination which is responsible for the generation of variability, would not be expected to be an effective source of high affinity and high specificity antibodies.

EP-B-0368684 (Medical Research Council) discloses the construction of expression libraries comprising a repertoire of nucleic acid sequences each encoding at least part of an immunoglobulin variable domain and the screening of the encoded domains for binding activities. It is stated that repertoires of genes encoding immunoglobulin variable domains are preferably prepared

from lymphocytes of animals immunised with an antigen. The preparation of antigen binding activities from single VH domain, the isolation of which is facilitated by immunisation, is exemplified (see Example 6). Repertoires of amplified heavy chain variable domains obtained from mouse immunised with lysozyme and from human peripheral blood lymphocytes were cloned into expression vectors and probed for lysozyme binding activity. It is reported that 2 positive clones (out of 200) were identified from the amplified mouse spleen DNA and 1 clone from the human cDNA. A library of VH domains from the immunised mouse was screened for lysozyme and keyhole limpet haemocyanin (KLH) binding activities; from 2000 colonies, 21 supernatants were found to have lysozyme binding activity and 2 to have KLH binding activity. An expression library prepared from a mouse immunised with KLH screened in the same manner gave 14 supernatants with KLH binding activity and only 1 with lysozyme binding activity. These results suggest to the Applicants that although antigen binding activities can be seen, these are of very low specificity and affinity (presumably due to the absence of the stabilising effect of the missing light chain such that only half of the designed binding pocket is present, leading to binding with related or homologous targets).

Immunoglobulins capable of exhibiting the functional properties of conventional (four-chain) immunoglobulins but which comprise two heavy polypeptide chains and which furthermore are devoid of light polypeptide chains have been described (see European Patent Application EP-A-0584421, Casterman et al, 1994). Fragments of such immunoglobulins, including fragments corresponding to isolated heavy chain variable domains or to heavy chain variable domain dimers linked by the hinge disulphide are also described. Methods for the preparation of such antibodies or fragments thereof on a large scale comprising transforming a mould or yeast with an expressible DNA sequence encoding the antibody or fragment are described in patent application WO 94/25591 (Unilever).

The immunoglobulins described in EP-A-0584421, which may be isolated from the serum of Camelids, do not rely upon the association of heavy and light chain variable domains for the formation of the antigen-binding site but instead the heavy polypeptide chains alone naturally form the complete antigen binding site. These immunoglobulins, hereinafter referred to as "heavy-chain immunoglobulins" are thus quite distinct from the heavy chains obtained by the degradation of conventional (four-chain) immunoglobulins or by direct cloning. Heavy chains from conventional immunoglobulins contribute part only of the antigen-binding site and require a light chain partner, forming a complete antigen binding site, for optimal antigen binding.

As described in EP-A-0584421, heavy chain immunoglobulin V_H regions isolated from Camelids (forming a complete antigen binding site and thus constituting a single domain binding site) differ from the V_H regions derived from conventional four-chain immunoglobulins in a number of respects, notably in that they have no requirement for special features for facilitating interaction with corresponding light chain domains. Thus, whereas in conventional (four-chain) immunoglobulins the amino acid residue at the positions involved in the V_H/V_L interaction is highly conserved and generally apolar leucine, in Camelid derived V_H domains this is replaced by a charged amino acid, generally arginine. It is thought that the presence of charged amino acids at this position contributes to increasing the solubility of the camelid derived V_H . A further difference which has been noted is that one of the CDRs of the heavy chain immunoglobulins of EP-A-0584421, the CDR₃, may contain an additional cysteine residue associated with a further additional cysteine residue elsewhere in the variable domain. It has been suggested that the establishment of a disulphide bond between the CDR₃ and the remaining regions of the variable domain could be important in binding antigens and may compensate for the absence of light chains.

cdna libraries composed of nucleotide sequences coding for a

heavy-chain immunoglobulin and methods for their preparation are disclosed in EP-A-0584421. It is stated that these immunoglobulins have undergone extensive maturation *in vivo* and the V region has naturally evolved to function in the absence of the light chain variable domain. It is further suggested that in order to allow for the selection of antibodies having specificity for a target antigen, the animal from which the cells used to prepare the library are obtained should be pre-immunised against the target antigen. No examples of the preparation of antibodies are given in the specification of EP-A-0584421. The need for prior immunisation is also referred to in Arabi Ghahroudi et al (FEBS Letters, 414 (1997), 521-526.

SUMMARY OF THE INVENTION

In a first aspect, the invention provides an expression library comprising a repertoire of nucleic acid sequences cloned from a non-immunised source, each nucleic acid sequence encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains. Further provided is a method of preparing a cDNA expression library as set forth above comprising providing a repertoire of mRNA from a non-immunised source, treating the obtained RNA with a reverse transcriptase to obtain the corresponding cDNA and cloning the cDNA, with or without prior PCR amplification, into an expression vector. Expression vectors comprising such nucleic acid sequences and host cells transformed with such expression vectors are also provided.

Further provided is the use of a non-immunised source of nucleic acid sequences encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains to prepare an expression library.

In another aspect, the invention provides a method for the preparation of antibody fragments derived from a non-immunised

source having specificity for a target antigen comprising screening an expression library as set forth above for antigen binding activity and recovering antibody fragments having the desired specificity.

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The invention further provides the use of a non-immunised source of nucleic acid sequences encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains to prepare an antibody, or fragment thereof, having binding specificity for a target antigen.

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According to a further aspect, nucleic acid sequences encoding antibody fragments isolated from such a repertoire of variable region genes may be attached to nucleic acid sequences encoding one or more suitable heavy chain constant domains and expressed in a host cell, providing complete heavy chain antibodies.

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By means of the invention, antibodies, particularly fragments thereof, having a specificity for a target antigen may conveniently be prepared by a method which does not require the donor previously to have been immunised with the target antigen. The method of the invention provides an advantageous alternative to hybridoma technology, or cloning from B cells and spleen cells where for each antigen, a new library is required.

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The present invention may be more fully understood with reference to the following description, when read together with the accompanying drawings.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of the domain structure of the 'classical' four-chain/two domain antibodies (a) and the camelid two chain/single domain antibodies (b).

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Figure 2 shows a plasmid map of phage display vector pHEN.5 containing a heavy chain variable domain (HC-V) gene. The DNA and protein sequences of the insertion regions are indicated.

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Figures 3A, 3B show a specificity ELISA assay of HC-V-myc samples of clones selected by panning on RR6-BSA (1% gelatin block).

A Specific clones.

B 'sticky' aspecific clones.

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RR-6 is an azo dye, available from ICI; BSA is bovine serum albumin; myc is a peptide comprising the sequence Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn.

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Figure 4 shows inhibition assays of HC-Vs selected by panning on RR6-BSA. Crude HC-V-myc samples were preincubated with increasing concentrations of RR6-BSA, followed by assay of free HC-V-myc on immobilised RR6-BSA.

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Figure 5 shows aligned protein sequences of selected anti-RR6 clones. The CDR regions are boxed.

Figure 6 shows a specificity ELISA assay of HC-V-myc samples of clones selected by panning on Dicarboxylic linoleic acid - ovalbumin conjugate (Di-OVA) (1% gelatin block).

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Figure 7 shows inhibition of antigen binding activity of the anti-dicarboxylic acid clones D1, D2 and D3 by the presence of free target antigen (Di-OVA) or control conjugate (estrone 3-glucuronide, E3G-OVA).

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Figure 8 shows aligned protein sequences of the three selected anti-dicarboxylic clones D1, D2, D3. The CDR regions are boxed.

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Figure 9 shows the effect of ammonium thiocyanate (ATC) on binding of HC-Vs to immobilised RR6-BSA. Increasing concentrations of ATC were added to crude HC-V-myc samples bound to immobilised RR6-BSA, followed by detection of remaining bound HC-V using anti-myc monoclonal antibody.

Figure 10 shows the effect of ATC on binding of HC-Vs to immobilised Di-OVA. Increasing concentrations of ATC were added to crude HC-V-myc samples bound to immobilised Di-OVA, followed by detection of remaining bound HC-V using anti-myc monoclonal antibody.

DETAILED DESCRIPTION OF THE INVENTION

The invention is based on the unexpected finding that highly specific antibody fragments against a target antigen may be provided by screening an expression library comprising a repertoire of nucleic acid sequences, each encoding at least part of a variable domain of a heavy chain derived from a non-immunised source of an immunoglobulin naturally devoid of light chains, for antigen binding activity. It would not be predicted that single domain libraries would provide high affinity/high specificity antibodies for the reasons of absence of combinatorial effect discussed above. From the teaching of EP-A-0584421, it would have been expected that in order to produce an antibody specific for a target antigen, either pre-immunisation of the donor with the target antigen or random combination with a VL domain would be necessary.

As used herein, the term "antibody" refers to an immunoglobulin which may be derived from natural sources or synthetically produced, in whole or in part. An "antibody fragment" is a portion of a whole antibody which retains the ability to exhibit antigen binding activity.

A "library" refers to a collection of nucleic acid sequences. The term "repertoire", again meaning a collection, is used to indicate genetic diversity.

5 The heavy chain variable domains for use according to the invention may be derived from any immunoglobulin naturally devoid of light chains, such that the antigen-binding capability and specificity is located exclusively in the heavy chain variable domain. Preferably, the heavy chain variable domains for use in
10 the invention are derived from immunoglobulins naturally devoid of light chains such as may be obtained from Camelids, as described in EP-A-0584421, discussed above.

Expression libraries according to the invention may be generated
15 using conventional techniques, as described, for example, in EP-B-0368684 and EP-A-0584421. Suitably, a cDNA library comprising a repertoire of nucleic acid sequences each encoding a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains may be generated by cloning cDNA from
20 lymphoid cells, with or without prior PCR amplification, into a suitable expression vector.

Preferably, the nucleic acid sequences used in the method according to the invention are derived from mRNA which may
25 suitably be isolated using known techniques from cells known to produce immunoglobulins naturally devoid of light chains. mRNA obtained in this way may be reacted with a reverse transcriptase to give the corresponding cDNA. Alternatively, the nucleic acid sequences may be derived from genomic DNA, suitably from
30 rearranged B cells.

Suitable sources of heavy chain variable domains derived from immunoglobulins naturally devoid of light chains include lymphoid cells, especially peripheral blood lymphocytes, bone marrow cells,
35 spleen cells derived from camelids.

The nucleic acid sequences encoding the heavy chain variable domains for use according to the invention are cloned into an appropriate expression vector which allows fusion with a surface protein. Suitable vectors which may be used are well known in the art and include any DNA molecule, capable of replication in a host organism, into which the nucleic acid sequence can be inserted. Examples include phage vectors (for example, lambda, T4), more particularly filamentous bacteriophage vectors such as M13. Alternatively, the cloning may be performed into plasmids, such as plasmids coding for bacterial membrane proteins or eukaryotic virus vectors.

The host may be prokaryotic or eukaryotic but is preferably bacterial, particularly *E. coli*.

If the cloned nucleic acid sequences are introduced into an expression vector containing nucleic acid sequences encoding one or more constant domains, heavy chain immunoglobulin chains may be expressed.

Preferably, the cloned nucleic acid sequences may be inserted in an expression vector for expression as a fusion protein.

The expression library according to the invention may be screened for antigen binding activity using conventional techniques well known in the art as described, for example, in Hoogenboom, Tibtech, 1997 (15), 62-70. By way of illustration, bacteriophage displaying a repertoire of nucleic acid sequences according to the invention on the surface of the phage may be screened against different antigens by a 'panning' process (see McCatterty, Nature, 348, (1990), 552-554) whereby the heavy chain variable domains are screened for binding to immobilised antigen. Binding phage are retained, eluted and amplified in bacteria. The panning cycle is repeated until enrichment of phage or antigen is observed and individual phage clones are then assayed for binding to the panning antigen and to uncoated polystyrene by phage ELISA.

Suitable antigens include RR-6 and di-carboxylic linoleic acid.

5 In accordance with a particular embodiment of the invention, the genes encoding the variable domains of the single domain antibodies of six individual Llamas (which had not been in contact with any of the later used antigens) were isolated and cloned into the phage display vector pHEN which allows the expression of active antibody fragments on the tip of the phage. Eleven
10 libraries (six 'long hinge' and five 'short hinge'), each containing about 10^6 individual members were constructed, together yielding a single 'one-pot' library of approximately 10^7 members with a very high level of complexity.

15 The library was screened for binding to RR-6 and Di-carboxylic linoleic acid using a panning process. After four and five rounds of panning a significant enrichment was observed for both antigens. After screening individual clones for specific binding activity to its antigen a large number of positive clones were
20 identified via ELISA. Using ELISA technique the clones were shown to be highly active and exhibited strong antigen specific recognition.

The following examples are provided by way of illustration only.
25 Techniques used for the manipulation and analysis of nucleic acid materials were performed as described in Sambrook et al, *Molecular Cloning*, Cold Spring Harbour Press, New York, 2nd Ed. (1989), unless otherwise indicated.

30 HC-V denotes heavy chain variable domain.

EXAMPLES**EXAMPLE 1. Construction of the naive HC-V library.****5 1.1 Isolation of gene fragments encoding llama HC-V domains**

A blood sample of about 200ml was taken from an non-immunised Llama and an enriched lymphocyte population was obtained via Ficoll (Pharmacia) discontinuous gradient centrifugation. From these cells, total RNA was isolated by acid guanidium thiocyanate
 10 extraction (e.g. via the method described by Chomczynski and Sacchi, (Anal. Biochem, 162, 156-159 (1987)). After first strand cDNA synthesis (e.g. with the Amersham first strand cDNA kit), DNA fragments encoding HC-V fragments and part of the long or short hinge region were amplified by PCR using specific primers:

*Pst*I

V_H - 2B 5'-AGGTSMAR**CTGCAGS**AGTCWGG-3'

(see SEQ. ID. NO: 1).

*Sfi*I

PCR.162:5'-

CATGCCATGACTCGCGGCCAGCCGGCCATGGCCSAGGTSMAR**CTGCAGS**AGTCWGG-3

(see SEQ. ID. NO: 2).

25 S =C and G, M = A and C, R = A and G , W =A and T,

*Hind*III *Not*I

Lam-07:5'-AACAGTT**AAGCTT**CCGCTTGCGGCCGCGGAGCTGGGGTCTTCGCTGTGGTGCG-3'

(see SEQ. ID. NO: 3).

*Hind*III *Not*I

30 Lam-08:5'-AACAGTT**AAGCTT**CCGCTTGCGGCCGCTGGTTGTGGTTTTGGTGTCTTGGGTT-3'

(see SEQ. ID. NO: 4).

35 Upon digestion of the PCR fragments with *Pst*I (coinciding with codon 4 and 5 of the HC-V domain, encoding the amino acids L-Q)

and NotI (located at the 3'-end of the HC-V gene fragments), the DNA fragments with a length between 300 and 400bp (encoding the HC-V domain, but lacking the first three and the last three codons) were purified via gel electrophoresis and isolation from the agarose gel. NotI has a recognition-site of 8 nucleotides and it is therefore not likely that this recognition-site is present in many of the created PCR fragments. However, PstI has a recognition-site of only 6 nucleotides. Theoretically this recognition-site could have been present in 10% of the created PCR fragments, and if this sequence is conserved in a certain class of antibody fragments, this group would not be represented in the library cloned as PstI-NotI fragments. Therefore, a second series of PCR was performed, in which the primary PCR product was used as a template (10ng/reaction). In this reaction the 5' VH2B primer was replaced by PCR162. This primer introduces a SfiI recognition-site (8 nucleotides) at the 5' end of the amplified fragments for cloning. Thus, a total of 24 different PCR products were obtained, four (short and long hinge, Pst I/Not I and Sfi I/Not I) from each Llama. Upon digestion of the PCR fragments with SfiI (upstream of the HC-V coding sequence, in the pelB leader sequence) and NotI, the DNA fragments with a length between 300 and 400bp (encoding the HC-V domain) were purified via gel electrophoresis and isolation from the agarose gel.

1.2 Construction of HCV Library in pHEN.5

The Pst I/Not I or Sfi I/Not I - digested fragments were purified from agarose and inserted into the appropriately digested pHEN.5 vector (Figure 2). Prior to transformation, the ligation reactions were purified by extraction with equal volumes of phenol/chloroform, followed by extraction with chloroform only. The DNA was precipitated by addition of 0.1 volume 3M NaAc pH5.2 and 3 volumes ethanol. The DNA pellets were washed x2 with 1ml 70% ethanol, dried and resuspended in 10 µl sterile milliQ water. Aliquots were transformed into electrocompetent *E.coli* XL1-Blue (Stratagene) by electroporation, using a Bio-Rad Gene Pulser. The protocol used was as recommended by Stratagene. The final

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library, consisting of approximately 7.8×10^6 individual clones, was harvested by scraping the colonies into 2TY + Ampicillin (100ug/ml) + Glucose (2% w/v) culture medium (35-50ml each). Glycerol stocks (30% v/v) and DNA stocks were prepared from these and stored at -80°C .

EXAMPLE 2. Selection of HC-V fragments which exhibit antigen binding affinity.

2.1 Panning of the library

Two 'antigens' were used for screening the naive phage-displayed HCV library;

Di acid-OVA (dicarboxylic linoleic acid-ovalbumin conjugate) and the azo-dye RR6 (available from ICI) conjugated to BSA (reactive red six-bovine serum albumin conjugate).

Phages displaying antibody fragments on their surface were obtained using the following protocol:

Phage rescue:

15mL 2TY/Ampicillin/Glucose was incubated with 100 μ L of a glycerol stock of the naive library culture. The culture was allowed to grow until log-phase ($A_{600} = 0.3-0.5$), at which point 4.5×10^9 pfu M13K07 helper phage were added. After infection for 30 minutes at 37°C (without shaking) the infected cells were spun down (5000 rpm for 10 minutes) and the pellet was resuspended in 200mL 2xTY/Ampicillin/Kan. After incubation with shaking at 37°C overnight, the culture was spun and the phages present in the supernatant were precipitated by adding 1/5 volume PEG/NaCl (20% Polyethylene glycol 8000, 2.5M NaCl). After incubation on ice-water for 1 hour the phage particles were pelleted by centrifugation at 8000 rpm for 30 minutes. The phage pellet was resuspended in 20mL water and re-precipitated by adding 4mL PEG/NaCl solution. After incubation in ice-water for 15 minutes the phage particles were pelleted by centrifugation at 5000 rpm

for 15 minutes and resuspended in 2mL PBST with 2% Marvel (milk powder; trade name) (plus 2% OVA for the Di acid-OVA tube and 2% BSA for the RR6-BSA tube).

5 Panning;

The PEG precipitated phages in PBST/2%Marvel (0.5ml) (plus 2% OVA for the Di acid-OVA tube and 2% BSA for the RR6-BSA tube) were added to Nunc-immunotubes (5mL) coated with 1ml Di acid-OVA conjugate (100µg/ml), 1ml RR6-BSA conjugate (100µg/ml) or a control tube. All tubes were blocked with PBST/2% Marvel) (plus 2% OVA for the Di acid-OVA tube and 2% BSA for the RR6-BSA tube) at 37°C for 1 hour before the phages were added. After incubation for 3-4 hours at room temperature, unbound phage were removed by washing the tube 20 times with PBS-T followed by 20 washes with PBS. The bound phages were eluted by adding 1mL elution buffer (0.1M HCL/glycine pH2.2/1mg/mL BSA). The elution mixture was neutralised with 60µL 2M Tris, and the eluted phages were added to 9mL log-phase E.coli XL-1 Blue. Also 4mL log-phase E.coli XL-1 Blue were added to the immunotube. After incubation at 37°C for 30 minutes to allow infection, the 10mL and 4mL infected XL-1 Blue bacteria were pooled and plated onto SOBAG plates (20g bacto-tryptone, 5g bacto-yeast extract, 0.1g Na Cl, 15g Agar; made up to 1 litre with distilled water and autoclaved, allowed to cool and 10mL MgCl₂ and 27.8 mL 2M glucose added. Following growth overnight at 37°C the clones obtained from the antigen sensitised tubes were harvested and used as starting material for the next round of panning, or alternatively individual colonies were assayed specific antigen binding activity.

30 For panning rounds 1 to 3 there was no indication of phage enrichment over background for both antigens (Table 1). However, at pan 4, significant enrichment of phages was observed for both RR6-BSA and Di-acid-OVA.

Table 1. Results of the panning reactions
(fold enrichment over background)

| Panning Antigen | Pan 1 | Pan 2 | Pan 3 | Pan 4 | Pan 5 |
|-----------------|-------|-------|-------|-----------|-------------|
| RR6 | none | none | none | 100-fold | ~200-fold |
| Di-acid | none | none | none | ~100-fold | 50-100-fold |

5 EXAMPLE 3. Identification of individual HC-V fragments with antigen binding activity.

Individual bacterial colonies were picked (200 from pans 4 and 5, for both antigens) using sterile toothpicks and added to the wells of 96-well microtitre plates (Sterilin) each containing 100ml of 2TY, 1% (w/v) glucose and ampicillin (100mg/ml). After allowing the cultures to grow overnight at 37°C, 20µl aliquots from each well of these 'masterplates' were added to the wells of fresh microtitre plates each containing 200ml of 2TY, 1% glucose, 100mg/ml ampicillin, 10⁹ M13KO7 helper phage. Infection at 37°C for 2.5h was followed by pelleting the cells and resuspending the infected cells in 200ml of 2TY containing ampicillin (100mg/ml) and kanamycin (25mg/ml). Following overnight incubation at 37°C, the phage-containing supernatants (100µl) were added to the wells of Sterilin microtitre plates containing 100µl/well of the appropriate blocking buffer (same buffer used as during panning reactions). Pre-blocking of the phage was carried out in these plates for 30 mins at room temp. After 30 minutes at room temperature, 100µl of phage supernatant was added to the wells of a Greiner HC ELISA plate coated with the corresponding antigen, and to the wells of an uncoated plate. After 2h incubation at 37°C unbound phages were removed, and bound phages were detected with rabbit anti-M13 followed a goat anti-rabbit alkaline phosphatase conjugate. The assays were developed with 100ml/well of p-nitrophenyl phosphate (1mg/ml) in 1M diethanolamine, 1mM MgCl₂, pH9.6 and the plates read after 5-10 mins at 410nm.

Table 2. Percentage of panned phage clones which specifically recognise and bind immobilised antigen.

| Panning Antigens | Pan 4 | Pan 5 |
|------------------|-------|-------|
| RR6-BSA | 23% | 43% |
| Diacid-OVA | 13% | 20% |

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EXAMPLE 4. Characterisation of HC-V fragments with specific RR-6 binding activity.

To test the individual clones identified in the phage ELISA's for their ability to produce active soluble antibody fragments, plasmid DNA from 12 clones that were shown to specifically recognise RR6-BSA was isolated and used to transform the non-suppressor E.coli strain D29AI. Commercially available strains such as TOPIOF (stratagene) and HB2151 (Pharmacia) may alternatively be used. Two transformants of each clone were pre-grown in 10ml 2TY/Ampicillin/Glucose. After 3-4 hours of growth at 37°C (OD₆₀₀=0.5), the cells were pelleted by centrifugation and resuspended in 5ml 2TY/Ampicillin/IPTG (0.1mM). After 24 hours of incubation at 25°C the cultures were centrifuged, and the supernatants were analysed for the production of antigen binding activity in essential the same way as described in Example 3. In this case, however, the presence of specifically bound HC-V fragments was detected by incubation with monoclonal anti-myc antibodies, followed by incubation with poly-clonal rabbit-anti-mouse conjugate with alkaline phosphatase.

As shown in Figure 3A, six (nR1, nR2, nR5, nR7, nR11 and nR12) out of the twelve chosen RR6-BSA - panned clones were specific for RR6-BSA, and did not bind to any of the other antigens tested. The specificity of these 6 clones was also confirmed in competition assays in which following the protocol outlined above, soluble RR6 or RR6-BSA conjugate was present during the antigen

binding reaction and was shown to reduce the specific binding signal (Figure 4). Another three clones (nR3, nR4 and nR8) were specific for RR6-BSA, but the signals observed were very low. These weak ELISA signals correlated with relatively poor signals in dot-blot experiments, indicating that these clones were poor producers of soluble fragment. This was confirmed by analysis of the supernatants on Western blots (Figure 3B). The remaining 3 clones (nR6, nR9 and nR10) gave significant signals over background on RR6-BSA, BSA and E3G-OVA (Figure 3A). It would appear that these three 'sticky' clones bind to immobilised proteins in general.

The sequence of the isolated anti-RR6 HC-V fragments are listed in Figure 5.

| | |
|------|--------------------|
| nR1 | (SEQ. ID. NO: 5). |
| nR4 | (SEQ. ID. NO: 6). |
| nR5 | (SEQ. ID. NO: 7). |
| nR8 | (SEQ. ID. NO: 8). |
| nR11 | (SEQ. ID. NO: 9). |
| nR12 | (SEQ. ID. NO: 10). |

EXAMPLE 5. Characterisation of HC-V fragments with specific Di-Carboxylic Acid binding activity.

To test the individual clones identified in the phage ELISA's for their ability to produce active soluble antibody fragments, plasmid DNA from 9 clones that were shown to specifically recognise Di Acid-OVA was isolated and used to transform the non-suppressor E.coli strain D29AI. Two transformants of each clone were pre-grown in 10ml 2TY/Ampicillin/Glucose. After 3-4 hours of growth at 37°C (OD₆₀₀=0.5), the cells were pelleted by centrifugation and resuspended in 5ml 2TY/Ampicillin/IPTG (0.1mM). After 24 hours of incubation at 25°C the cultures were centrifuged, and the supernatants were analysed for the production of antigen binding activity in essential the same way as described

in Example 3. In this case, however, 1% gelatin was used as the blocking reagent and the presence of specifically bound HC-V fragments was detected by incubation with monoclonal anti-myc antibodies, followed by incubation with poly-clonal rabbit-anti-mouse conjugate with alkaline phosphatase.

3 of the selected HC-V samples gave high signals against Di acid conjugated to OVA, BSA or PTG (porcine thyro globulin), and background signals against all other immobilised antigens tested (Figure 6). Much lower signals for Di acid-OVA were observed for a further 2 clones (Figure 6). The specificity of the 3 leading clones was further demonstrated using competition assays as described in Example 4, which showed strong inhibition of Di-Acid-OVA binding of these clones when supernatants were preincubated with Di acid-OVA conjugate, whereas the same concentration range of the E3G-OVA conjugate had no inhibitory effect (Figure 7).

The sequence of the isolated anti-Di Acid HC-V fragments are listed in Figure 8.

| | |
|-----|--------------------|
| nD1 | (SEQ. ID. NO: 11). |
| nD2 | (SEQ. ID. NO: 12). |
| nD3 | (SEQ. ID. NO: 13). |